BACKGROUND OF THE INVENTION (Original)

This invention pertains to methods of quantitative analysis of alcohols in a sample by isotope dilution mass spectrometry . The stable isotope labeled esters and carbamates are used as internal standards. The sample may be a biological fluid, such as serum, urine etc., or an aqueous sample such as an environmental or an agricultural sample.

While various methods of analysis such as immunoassays and chromatographic analysis - LC (liquid chromatography), GC (gas chromatography), and TLC (thin layer chromatography) - have been reported for identification and determination of levels of alcohols in analytical samples, the absolute and unequivocal identification and quantitative analysis of those compounds are combinations of chromatographic analysis and MS (mass spectrometry) such as GC-MS and LC-MS. The accuracy and precision of these methods are usually the highest when stable isotope analogs of the analytes are used as internal standards. The mass spectrometry method of analysis using stable isotope internal standards is commonly called isotope dilution mass spectrometry. This method takes advantage of the similar chemical and physical behaviors of analytes and their respective isotope labeled internal standards towards all phases of sample preparation and also towards instrument responses. It uses the mass differentiation between analytes and their respective internal standard in mass spectrometry for quantification. The requirement for this method of analysis is the availability of stable isotope labeled internal standards.

The commonly used stable isotope labeled internal standard of an analyte is a chemical compound that has the same chemical structure as that of the analyte except that one or more substituent atoms are stable isotopes. Four commonly used stable isotopes are deuterium, carbon-

13, nitrogen-15, and oxygen-18. For every hydrogen atom that is replaced by a deuterium atom, the molecular weight of resulting chemical compound is increased by one mass unit. This is also true for replacing a carbon atom with a carbon-13 atom, or by replacing a nitrogen atom with a nitrogen-15 atom. In the case of replacing an oxygen atom with an oxygen-18 atom, the molecular increase is two mass units. Although the acceptable stable isotope labeled internal standard for isotope dilution mass spectrometry method is the one that is not contaminated with any of the unlabeled material, the ideal one should be the one with the highest isotopic purity and contains as many stable isotope atoms as possible. The ideal one, however, must not contain any labeled isotope that can be exchanged for the unlabeled isotope under particular sample preparation conditions.

These criteria of an ideal stable isotope labeled internal standard present a challenge for organic synthesis chemists who help the analytical chemists in the analysis. Most often the synthesis of stable isotope internal standards is not simply an isotope exchange reaction. Easily exchangeable atoms are usually avoided due to possible re-exchange during sample preparation steps. Organic chemists often have to carry out multi-step synthesis to make stable isotope labeled internal standards. Even though many stable isotope labeled reagents are commercially available, the choice of appropriate labeled reagent for chemical synthesis of stable isotope labeled internal standards is still very limited. The limited isotope labeled reagents and the multi-step synthesis contribute to the high cost of synthesis of stable isotope internal standards. Even if the analytical chemist who carries out the analysis can afford the cost of the synthesis, there is also a time factor that he or she has to consider before ordering the synthesis. Situations where organic chemists spent weeks and months on a synthesis project and came up with nothing at the end were common. This invention offers a solution for this problem.

The objective is a short and reliable method of preparing a stable isotope labeled internal standard that is suitable for the analysis of an analyte in question, but not the synthesis of the stable isotope labeled analyte. Within the context of the isotope dilution mass spectrometry method, both analyte and its internal standard have to have identical chemical structures, with the exception of the isotope atoms which provide the mass differentiation upon mass spectrometric analysis. Analytical chemists who uses GC-MS for their analysis often "derivatize" the analyte and its stable isotope labeled analyte (used as internal standard) into chemical compounds that can easily pass through the GC column or else provide better instrumental responses. The analysis becomes the analysis of the"derivatized" analyte and the "derivatized" internal standard, but still provides comparably accurate results of concentrations of the analyte itself. Examples of these analyses are found in cited references. Using similar reasoning, one can synthesize a stable isotope derivative of the analyte by reacting it with a stable isotope labeled reagent. The resulting isotope labeled chemical compound can be used as internal standard in the analysis of the analyte, providing that the analyte in the analyzed sample will be converted to a chemical compound of identical structure as that of the internal standard using a non-labeled reagent. There are 3 requirements for the usefulness of this method :

1. The analyte in the sample must be ***quantitatively*** converted to the compound of identical structure (except the labeled atoms) as that of the added isotope labeled internal standard using a non-labeled reagent.
2. Absolutely no conversion of the isotope labeled internal standard to the non-labeled compound because the conversion of the analyte happens in the sample in the presence of the added isotope labeled internal standard.
3. The conversion of the analyte into the compound of identical structure as that of the added

isotope labeled internal standard has to be accomplished before any isolation method i.e. extraction, is performed.

The first two requirements relate to the chemistry of the analyte in question. The efficiency of a chosen chemical reaction depends on the type of reaction which, in turn, depends on the type of functional groups of the analyte. This invented method relates to the analysis of alcohols whose chemistry focus on the reactivity of the hydroxyl functional groups of the analytes.

Quantitative reactions of alcohols in aqueous samples are :

1. Conversion to an ester using an acid anhydride or an acid chloride.
2. Conversion to a carbamate using an isocyanate.

There are other reactions of alcohols that are very efficient, but the above conversion reactions are very efficient in aqueous environment and can be performed at room temperature and in a relatively short reaction time. These are necessary and practical features for routine analysis of alcohols in aqueous samples.

BRIEF SUMMARY OF THE INVENTION (Original)

The current invention provides for a method of identification and quantification of alcohol in a sample by isotope dilution mass spectrometry . The stable isotope labeled internal standard of said alcohol is synthesized beforehand by reacting a sample containing said analyzed alcohol with a labeled reagent. Following this step, said stable isotope labeled internal standard is then added to a sample containing said analyzed alcohol. Said analyzed alcohol is then converted to a non-labeled analog of said labeled internal standard with identical chemical structure as said labeled internal standard except for the stable isotope atoms using a non-labeled reagent. Both said converted analyzed alcohol and said stable isotope labeled internal standard are then extracted and analyzed by mass spectrometry. Said stable isotope labeled internal standards provided in the current invention are labeled ester and carbamate analogs of said analyzed alcohol. The type of labeled internal standard used will dictate said labeled reagents used for its synthesis as well as said non-labeled reagent used to convert the analyzed alcohol to the corresponding analog.

In comparison with the traditional method of isotope dilution mass spectrometric analysis of more than one alcohols, the invented method offers the following advantages :

1. The efficiency and simplicity of the above reactions makes possible the short, reliable, and quick synthesis of individual stable isotope labeled internal standards, whereas in the traditional method of analysis, stable isotope labeled internal standard of each alcohol has to be independently synthesized.
2. It is possible to quickly and efficiently synthesize a library of stable isotope internal standards for the analysis of an entire library of alcohols using these reactions and only one commercially available stable isotope labeled reagent.

3. Because the synthesis of stable isotope labeled internal standard in this invented method is usually a one-step synthesis, the entire process of synthesis and sample preparation can be performed in an automated fashion. The internal standard is prepared in one step, excess isotope reagent is then destroyed, and the prepared internal standard can be added directly to the samples without purification. The non-labeled reagent is added and the sample is ready for extraction shortly thereafter.

These attractive features make the method suitable for high throughput analysis of alcohols by isotope dilution mass spectrometry.

DETAILED DESCRIPTION OF THE INVENTION (New and Original)

The current invention provides for a method of identification and quantification of alcohol(s) in a sample by mass spectrometry . Said alcohol(s) has the following formulas R_1OH , R_1CH_2OH , R_1R_2CHOH , $R_1R_2R_3COH$ wherein R_1, R_2, and R_3 are alkyl, aryl, and heteroatom containing cyclic or non-cyclic groups. The current method comprises, as an intergral part of the analysis of said alcohol(s), the following steps :

1. Synthesizing labeled ester internal standard(s) by reacting an authentic sample of said alcohol(s) with a stable isotope labeled reagent to form said ester internal standard(s) of the general formulas R_1OCOR_4 or $R_1CH_2OCOR_4$ or $R_1R_2CHOCOR_4$ or $R_1R_2R_3COCOR_4$, wherein R_4 is a stable isotope labeled alkyl or aryl group. Said R_4 stable isotope labeled alkyl or aryl group is selected from the group consisting of CD_3, CD_2CD_3 or C_6D_5. Said stable isotope labeled reagent is a labeled acid anhydride or an acid chloride selected from a group consisting of labeled acetic acid anhydride, labeled propionic acid anhydride and labeled benzoic acid anhydride or labeled acetyl chloride, labeled propionyl chloride, and labeled benzoyl chloride.
2. A known amount of said stable isotope labeled ester internal standard(s) was then added to said sample containing said alcohol(s) to be analyzed.
3. Said sample was then contacted with a non-labeled acid anhydride or an acid chloride selected from a group consisting of acetic acid anhydride, propionic acid anhydride and benzoic acid anhydride or acetyl chloride, propionyl chloride, and benzoyl chloride to quantitatively convert said alcohol(s) in said sample into said ester(s) of identical structure as that of said ester internal standard(s) except for the stable isotope atoms.

4. Appropriate extraction methods were then used to isolate said ester(s) and their corresponding ester internal standard(s) from said sample. Concentration of said ester(s) were determined and quantified by mass spectrometry and based on the ratio of said converted ester(s) and their corresponding ester internal standard(s).

In another aspect of the present invention, said labeled internal standard is a stable isotope labeled carbamate. In this embodiment, said stable isotope labeled carbamate(s) is synthesized by reacting an authentic sample of said alcohol(s) with a stable isotope labeled reagent to form said carbamate internal standard(s) having the following formula R_1OCONR_4 or $R_1CH_2OCONR_4$ or $R_1R_2CHOCONR_4$ or $R_1R_2R_3COCONR_4$ wherein R_4 is a stable isotope labeled alkyl or aryl group selected from the group consisting of CD_3, CD_2CD_3, C_6D_5. Said stable isotope labeled reagent is a labeled isocyanate selected from a group consisting of labeled methyl isocyanate, labeled ethyl isocyanate and labeled phenyl isocyanate. Also, in this embodiment, said analyzed alcohol(s) is converted to carbamate(s) of identical structure as that of said carbamate internal standard(s) except for the stable isotope atoms by contacting said sample with a non-labeled isocyanate selected from a group consisting of methyl isocyanate, ethyl isocyanate and phenyl isocyanate.

Example : Analysis of Naltrexone in human plasma.

Step 1 : Preparation of Naltrexone acetate ester-d3.

A solution of 2.5 mg of Naltrexone in 0.5ml aqueous sodium bicarbonate was treated with 5 equivalents of acetic anhydride-d6. The resulting solution was stirred for 2 hours then the aqueous phase was extracted with ethyl acetate-hexane mixture and the combined organic phases were dried with magnesium sulfate. The filtered solution was concentrated and the residue was purified by column chromatography using silica gel as absorbant and hexane ethyl acetate

mixture as eluant. The fractions containing clean naltrexone acetate ester-d3 were combined and concentrated to give 0.5mg product as an oil. MS analysis gave MH+ 387.

Step 2 : Preparation of working standard solutions and internal standard solution.

Working standard solutions of naltrexone were prepared by weighing naltrexone and diluting the stock solution to appropriate concentration as follows :

Solution A	0.5 ng/ml in ethyl acetate
B	1 ng/ml
C	2 ng/ml
D	5 ng/ml
E	25 ng/ml
F	100 ng/ml
G	125 ng/ml

Working quality control standard solutions of naltrexone were prepared by independently weighing naltrexone and diluting the stock solution to appropriate concentration as follows :

QC Solution J	1.5 ng/ml in ethyl acetate
K	70 ng/ml

Working internal standard solution of naltrexone were prepared by preparing a stock solution of naltrexone acetate ester-d3 and diluting the stock solution to a working concentration of 10 ng/ml in ethyl acetate.

Step 3 : Preparation of calibration samples and quality control samples in human plasma.

Naltrexone-free human plasma aliquots of 0.1ml were treated with 1000ul of solution A to G to make calibration samples A to G.

Naltrexone -free human plasma aliquots of 0.1ml were treated with 1000ul of solution J and K to make quality control samples J and K.

Both calibration samples and quality control samples were then treated with 400ul of the internal standard working solution.

Step 4 : Conversion to esters and extraction.

To all prepared samples were added 100ul of 1M aqueous sodium bicarbonate followed by 100ul of a 10% v/v acetic anhydride in ethyl acetate. The samples were mixed and shaked at room temperature for 15 minutes. The samples were extracted with 0.5ml ethyl acetate. Each extract was separated and concentrated. The residue of each extract was reconstituted with 100ul of acetonitrile.

Step 5 : Analysis of reconstituted extracts by LC/MS/MS.

A total of 9 reconstituted extracts were loaded on a Perkin Elmer autosampler that was connected to aPerkin Elmer LC pump and a PE Sciex API 365 MS. Each extract was run through an Symmetry C-18 column of 5um at a rate of 0.3ml/min of acetonitrile/water 50/50 mixture.

The eluate was directly fed to the MS ion source. MS data were collected for 1.5min per injection.

MS analysis was performed in MRM mode. m/z 384.0 > m/z 366.2 was monitored for naltrexone acetate ester while m/z 387.0 > m/z 369.2 was monitored for naltrexone acetate ester-d3. Collected data were ploted against concentration using McQuan 1.5 sofware.
Results are tabulated as follows:
Naltrexone
Internal Standard: is

Weighted (1/x*x)
Intercept = 0.037
Slope = 0.125
Correlation Coeff. = 0.994
Use Area

Filename	Filetype	Accuracy	Conc.	Calc. Conc.	Int. Ratio
Keto A	Standard	95.797	0.500	0.479	0.079
Keto B	Standard	99.105	1.000	0.991	0.160
Keto C	Standard	113.326	2.000	2.267	0.319
Keto D	Standard	114.829	5.000	5.741	0.752
Keto E	Standard	96.193	25.000	24.048	3.032
Keto F	Standard	89.559	100.000	89.559	11.193
Keto G	Standard	92.888	125.000	116.110	14.500
Keto J	QC	87.039	1.500	1.306	0.200
Keto K	QC	126.069	70.000	88.248	11.029